

The HIV Tat protein transduction domain improves the biodistribution of β -glucuronidase expressed from recombinant viral vectors

Haibin Xia^{1†}, Qinwen Mao^{1†}, and Beverly L. Davidson^{1-3*}

Treatment of inherited genetic diseases of the brain remains an intractable problem. Methods to improve the distribution of enzymes that are injected or expressed from transduced cells will be required for many human brain therapies. Recent studies showed that a peptide, the protein transduction domain (PTD) from HIV Tat, could improve the distribution of cytoplasmic reporter proteins when administered systemically as fusion proteins or cross-linked chimeras. The utility of this motif for noncytoplasmic proteins has not been determined. Here, we tested how the Tat motif affected uptake and biodistribution of the lysosomal enzyme β -glucuronidase, the protein deficient in the disease mucopolysaccharidosis VII, when expressed from viral vectors. The Tat motif allowed for mannose-6-phosphate (M6P) independent uptake *in vitro* and significantly increased the distribution of β -glucuronidase secreted from transduced cells after intravenous or direct brain injection in mice of recombinant vectors. Thus, enzymes modified to contain protein transduction motifs may represent a general strategy for improving the distribution of secreted proteins following *in vivo* gene transfer.

Collectively, the prevalence of lysosomal storage diseases is strikingly high. As an example, a 16-year retrospective study in Australia revealed a prevalence of between 1 in 6,700 to 1 in 7,700 live births¹. In 58% of cases, there was significant CNS involvement. Early work in rodent models of human lysosomal storage diseases has shown promise for addressing the systemic manifestations of these disorders, by either enzyme replacement or bone marrow transplant. However, these approaches did not ameliorate or substantially delay progressive neurodegeneration. In the β -glucuronidase-deficient mouse, an animal model of lysosomal storage disease with CNS involvement, inhibition of cognitive decline required either that systemic treatment be initiated in the neonatal period before blood-brain barrier closure² or that therapies be delivered directly to brain³.

Recent work showed that an 11-amino acid motif from HIV Tat, the protein transduction domain⁴, allows entry of nanoparticles into cells⁵ and improves the biodistribution of recombinant reporter proteins after systemic delivery^{4,6}. When partially denatured, the chimeric protein is capable of crossing the blood-brain barrier of adult mice⁴. These findings suggest that gene therapy with vectors engineered to express Tat-modified recombinant lysosomal proteins from systemic sources *in vivo* could be used to improve their biodistribution.

Results and discussion

To test this hypothesis, we engineered fusion proteins of human β -glucuronidase and the 11-amino acid protein transduction motif from HIV Tat in recombinant adenovirus expression vectors (Fig. 1A). Because peptides from the homeodomain of *Drosophila antennapedia* can translocate across cell membranes in either orientation⁷, the HIV Tat peptide was inserted in both the 47–57 and 57–47 orientations.

Native β -glucuronidase enters cells through the M6P receptor. We first examined the properties of the modified β -glucuronidase for M6P-dependent and -independent entry into cells. HeLa cells were

infected with 20 infectious units (i.u.) per cell of recombinant vectors expressing unmodified β -glucuronidase (Ad β gluc), β -glucuronidase–Tat_{47–57} (Ad β gluc–Tat_{47–57}), or β -glucuronidase–Tat_{57–47} (Ad β gluc–Tat_{57–47}). Three days later, supernatants were collected, and β -glucuronidase activity was quantified. The Tat modification to the C terminus did not inhibit enzyme activity. Equivalent units of the enzymes β -glucuronidase, β -glucuronidase–Tat_{47–57}, or β -glucuronidase–Tat_{57–47} were added to the media of A549 cells in the presence or absence of M6P (Fig. 1B–G). Although all recombinant proteins entered cells readily (Fig. 1B–D), M6P significantly inhibited the uptake of native β -glucuronidase (Fig. 1E) compared with β -glucuronidase–Tat_{47–57} or β -glucuronidase–Tat_{57–47} (Fig. 1F,G) as assayed by an *in situ* activity stain⁸. Quantitation of enzyme activity showed that M6P inhibited 100% of uptake of native β -glucuronidase. β -Glucuronidase–Tat_{47–57} and β -glucuronidase–Tat_{57–47} were inhibited by 20% and 38%, respectively (Fig. 1H). Thus, β -glucuronidase modified at the C terminus with the PTD of Tat allowed for both M6P-dependent and -independent entry. Similar results were found when supernatants containing the wild-type and Tat-modified β -glucuronidase were added to cultures of NIH3T3 cells with or without M6P (data not shown).

Earlier studies showed that uptake of Tat-modified proteins occurs by adsorptive endocytosis in cell lines and primary cell cultures^{6,9}. Mann and Frankel also showed that entry of ¹²⁵I-labeled Tat is temperature dependent⁹. This is in contrast to peptides from the antennapedia homeodomain, which enter cells readily at 4°C and 37°C (ref. 7). We added equivalent units of β -glucuronidase, β -glucuronidase–Tat_{47–57}, or β -glucuronidase–Tat_{57–47} to cells and compared uptake at 4°C and 37°C. In all cases, enzyme uptake at 4°C was significantly inhibited compared with that occurring at 37°C (data not shown). These data, and the observation that histochemical staining for enzyme activity at time points early after enzyme addition was punctate (Fig. 1D, inset), suggest that Tat-modified β -glucuronidase, like native β -glucuronidase, enters cells, in part,

¹Departments of Internal Medicine, ²Neurology, and ³Physiology and Biophysics, Program in Gene Therapy, University of Iowa College of Medicine, Iowa City, IA 52242 USA. *Corresponding author (beverly-davidson@uiowa.edu). †These two authors contributed equally to this work.

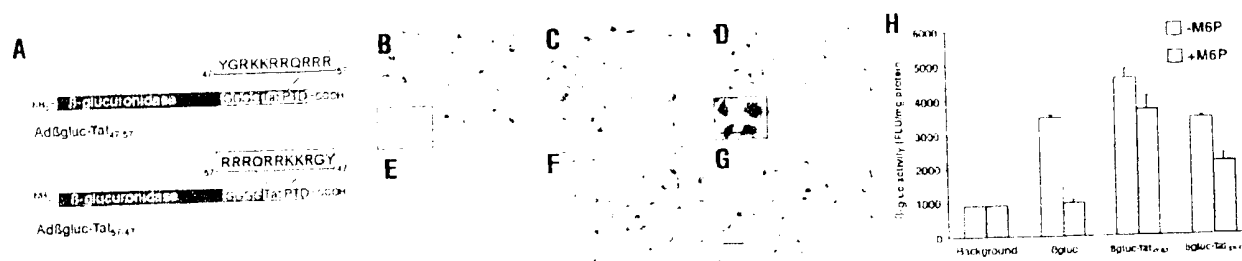


Figure 1. β-Glucuronidase-Tat expression vectors and recombinant protein uptake. (A) Cartoon depicting the orientation of the Tat motifs at the C termini of β-glucuronidase. Sequences encoding the β-glucuronidase fusion proteins were cloned into the E1 region of Ad shuttle plasmids, and the shuttles were recombined with Ad backbones expressing eGFP in the E3 region. The resultant viruses expressed β-glucuronidase, β-glucuronidase-Tat₄₇₋₅₇, or β-glucuronidase-Tat₅₇₋₄₇ in E1 and eGFP in E3. Both transgenes are driven off the Rous sarcoma virus (RSV) promoter. (B–D) β-Glucuronidase activity after incubation of A549 cells with the recombinant proteins β-glucuronidase, β-glucuronidase-Tat₄₇₋₅₇, and β-glucuronidase-Tat₅₇₋₄₇, respectively. Using the assay conditions described (see Experimental Protocol), we found that the background levels of β-glucuronidase staining were very low (inset in panel B). The uptake of both native (data not shown) and Tat-modified β-glucuronidase (inset in panel D) was notably punctate. (E–G) β-Glucuronidase activity after incubation of A549 cells with the recombinant proteins β-glucuronidase-Tat₄₇₋₅₇ (F), or β-glucuronidase-Tat₅₇₋₄₇ (G) in the presence of M6P. In panels B–H, bars = 50 μm. (H) Quantitation of enzyme activity in A549 cell lysates after incubation with recombinant β-glucuronidase, β-glucuronidase-Tat₄₇₋₅₇, or β-glucuronidase-Tat₅₇₋₄₇ in the presence or absence of M6P. M6P significantly reduced the uptake of all enzymes ($P < 0.005$). FLU, Fluorometric units.

through endocytic mechanisms.

We next investigated Adβgluc, Adβgluc-Tat₄₇₋₅₇, and Adβgluc-Tat₅₇₋₄₇ *in vivo*. Viruses were injected into mice tail veins, which results in transduction of hepatocytes¹⁰. The vectors used in this study also expressed green fluorescent protein (GFP) in the E3 region (see Experimental Protocol) to permit detection of infected cells (GFP positive) relative to β-glucuronidase, β-glucuronidase-Tat₄₇₋₅₇, or β-glucuronidase-Tat₅₇₋₄₇ activity. Sections of liver analyzed 10 days after intravenous vector injection show roughly equivalent levels of GFP expression for all viruses (Fig. 2A–C), but varied distribution of β-glucuronidase activity (Fig. 2D–F). β-Glucuronidase-Tat₄₇₋₅₇ and β-glucuronidase-Tat₅₇₋₄₇ activity were detected throughout the parenchyma of the liver as evidenced by *in situ* enzyme activity assay (Fig. 2E,F). In contrast, transduction with Adβgluc resulted in focal staining (Fig. 2D).

As in an earlier study¹⁰, β-glucuronidase, β-glucuronidase-Tat₄₇₋₅₇, and β-glucuronidase-Tat₅₇₋₄₇ activity was detected in tissues other than the liver (Fig. 3) after tail vein injection of the viruses. In some tissues, the penetration of the Tat-modified enzymes was remarkably different from that of native β-glucuronidase. For example, in the spleen of Adβgluc-injected mice (Fig. 3A), extensive β-glucuronidase activity was found in the marginal zone and to a limited extent in the red pulp. However, β-glucuronidase-Tat₄₇₋₅₇ was seen throughout the red pulp (Fig. 3B), as was β-glucuronidase-Tat₅₇₋₄₇ (data not shown). Interestingly, β-glucuronidase-Tat₄₇₋₅₇ distribution was similar to that found when mice were injected intraperitoneally with partially denatured, purified *Escherichia coli* β-galactosidase-Tat fusion proteins⁴.

We also noted increased levels of enzyme in kidney (Fig. 3C,D), lung (Fig. 3E,F), heart (Fig. 3G,H), and skeletal muscle (data not shown) from animals injected with recombinant viruses expressing

β-glucuronidase-Tat₄₇₋₅₇ and β-glucuronidase-Tat₅₇₋₄₇ (data not shown) relative to β-glucuronidase. Although the distribution of β-glucuronidase activity was widespread in kidney and lung in Adβgluc-Tat₄₇₋₅₇ versus Adβgluc-treated mice, β-glucuronidase activity remained undetectable in both lung lavage fluid and urine. Whether the increased distribution of β-glucuronidase within peripheral tissues after intravenous delivery of vectors resulted from increased secretion from transduced cells or from improved uptake as a consequence of the Tat motif remains unclear.

We noted only a modest increase in enzyme staining in brain, limited to the choroid plexus (Fig. 3I,J). Quantitative enzyme assay of brain lysates indicated that there were no significant differences between the treatment groups (Fig. 3K). Our results are similar to prior work by Fawell and colleagues⁶ using β-galactosidase-Tat conjugates given intraperitoneally but contrast with those described by Dowdy and coworkers⁴, in which denatured/partially renatured β-galactosidase-Tat fusion proteins were shown to cross the blood-brain barrier after intraperitoneal delivery. In both earlier reports^{4,6}, approximately 4×10^{-9} mol of β-galactosidase (estimated maximal serum concentration of 1 μM) was administered. In our studies, the Tat-modified β-glucuronidase reached an approximate serum concentration of 16 nM and likely remained in native conformation.

Our *in vitro* studies suggested that a proportion of the Tat-modified β-glucuronidase reached endocytic compartments (Fig. 1). Further studies in β-glucuronidase-deficient mice indicated that enzymatically active β-glucuronidase-Tat₄₇₋₅₇ was present in the lysosome. In four-week-old β-glucuronidase-deficient mice, significant storage pathology was evident within the red and white pulp of the spleen (Fig. 3L). Eighteen days after tail vein injection of Adβgluc-Tat₄₇₋₅₇ into age-matched mice, the storage pathology was cleared (Fig. 3M).

It is not known whether the partially denatured, Tat-modified reporter proteins described by Dowdy and colleagues can pass an

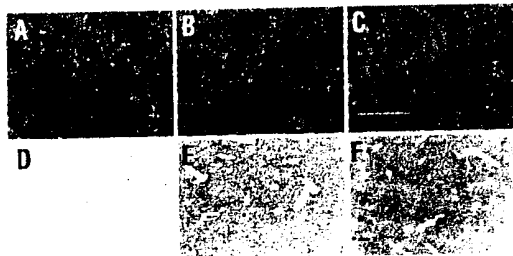


Figure 2. Activity of eGFP and β-glucuronidase in sections of murine liver after intravenous injection of vectors expressing native or Tat-modified β-glucuronidase. (A–C) Representative photomicrographs showing GFP expression levels in murine liver after injection of Adβgluc, Adβgluc-Tat₄₇₋₅₇, and Adβgluc-Tat₅₇₋₄₇, respectively. (D–F) Photomicrographs of representative sections from mice transduced with Adβgluc, Adβgluc-Tat₄₇₋₅₇, and Adβgluc-Tat₅₇₋₄₇, respectively, stained *in situ* for β-glucuronidase activity. Bars, 200 μm.

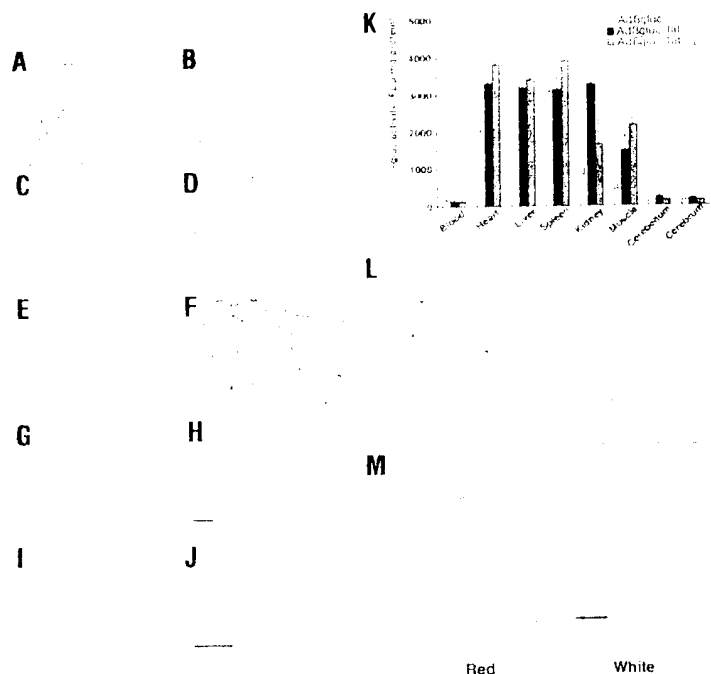


Figure 3. β-Glucuronidase activity in nonhepatic tissues after intravenous injection of mice with vectors expressing native or Tat-modified β-glucuronidase. β-Glucuronidase activity was detected *in situ* 10 days after intravenous injection of Adβgluc (A,C,E,G,I) or Adβgluc-Tat₄₇₋₅₇ (B,D,F,H,J). Representative sections from spleen (A,B), kidney (C,D), lung (E,F), heart (G,H), and brain (I,J) are shown. Scale bar is 400 μm for panels A–J. (K) Enzyme activity levels in tissue lysates. Enzyme activity in Adβgluc-Tat₄₇₋₅₇ or Adβgluc-Tat₅₇₋₄₇ injected mice was significantly higher in heart ($P < 0.05$ for both) and muscle ($P < 0.05$ for both) relative to Adβgluc injected mice. (L) Representative section of spleen from a β-glucuronidase-deficient mouse demonstrating the extensive lysosomal pathology in the red (left) and white (right) pulp by four weeks of age. (M) Intravenous injection of Adβgluc-Tat₄₇₋₅₇ drastically reduced the lysosomal distension. Bar, 10 μm.

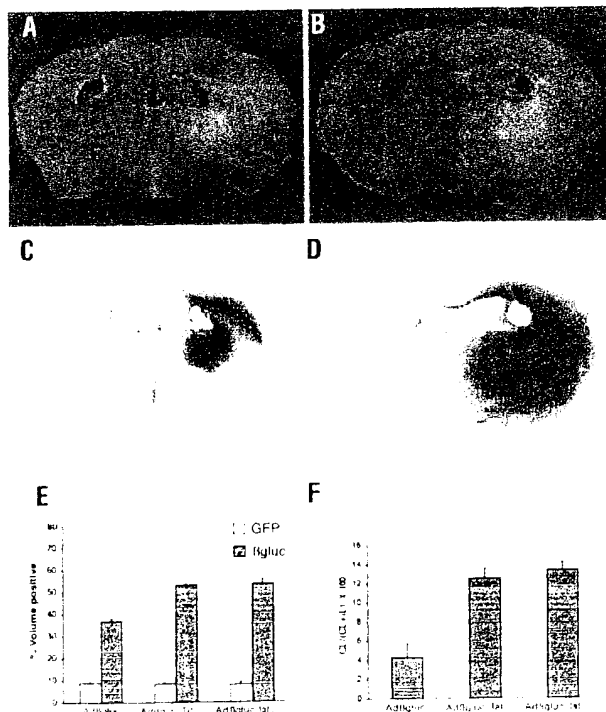


Figure 4. Distribution and activity of GFP and β-glucuronidase in brain. Mice were injected with Adβgluc (A,C), Adβgluc-Tat₄₇₋₅₇ (B,D), or Adβgluc-Tat₅₇₋₄₇ (data not shown) into striatum, and GFP and β-glucuronidase activity were evaluated 10 days later on full coronal sections (A–D) or tissue lysates (E,F). Equivalent i.u. (and particles) were injected. Sections photomicrographed in panels C and D are within 60 μm from those shown in panels A and B, respectively. (E) Volume of brain positive for GFP and β-glucuronidase. (F) Enzyme activities in tissue lysates of the contralateral (CL) and injected hemispheres (IL) were determined as described in the Experimental Protocol. The data are expressed as CL/(CL + IL) × 100.

intact blood–brain barrier in larger animal models. It also would be important to know whether the Tat motifs could improve distribution of proteins when administered directly to the brain or expressed from cells within the brain. To determine this, we injected vectors expressing β-glucuronidase, β-glucuronidase–Tat₄₇₋₅₇, or β-glucuronidase–Tat₅₇₋₄₇ (2×10^7 i.u.) into the right hemispheres of mice. The animals were killed 10 days later. All vectors yielded similar levels of GFP expression (Fig. 4A,B). β-Glucuronidase activity in brain lysates also were not significantly different (i.u./mg total protein was $5,678 \pm 138$, $5,817 \pm 159$, and $6,068 \pm 159$, for Adβgluc, Adβgluc-Tat₄₇₋₅₇, and Adβgluc-Tat₅₇₋₄₇ injected brains, respectively, $P = 0.25$). However, the addition of the Tat motif to β-glucuronidase resulted in a greater distribution of enzyme compared with the nonmodified protein (compare Fig. 4C vs. 4D). This resulted in a significant, 1.5-fold increase in the volume of brain positive for β-glucuronidase activity ($P < 0.0001$ for Adβgluc-Tat₄₇₋₅₇ and Adβgluc-Tat₅₇₋₄₇ injected brains vs. Adβgluc; Fig. 4E) and much greater levels of β-glucuronidase activity in the contralateral hemisphere ($P < 0.0001$; Fig. 4F). The altered distribution of enzyme in brain may be due to decreased rapid uptake through the M6P receptor or to enhanced secretion from transduced cells.

For the MPS or other lysosomal storage diseases, ventricular administration of secreted proteins or vectors expressing those proteins would be preferable to multiple parenchymal injections if adequate spread of enzyme into the parenchyma can occur. To test the effect of the Tat motif on the distribution of enzyme expressed from adenovirally transduced ependyma¹¹, we injected the recombinant vectors into the right lateral ventricles of mice. All viruses transduced the ependyma as evidenced by GFP fluorescence (Fig. 5A–C). As shown earlier¹¹, β-glucuronidase expression from Adβgluc was confined to areas immediately adjacent to the ependyma (Fig. 5D). However, the Tat modification enhanced the penetration of β-glucuronidase into the brain, significantly increasing the volume of cerebrum positive for active enzyme (Fig. 5E–G) and reducing the storage material in the cortex contralateral to the injection site (Fig. 5H,I). In animals receiving intraventricular injection of Adβgluc, ~4.5% of the brain was β-glucuronidase positive. In contrast, expression of β-glucuronidase–Tat₄₇₋₅₇ or β-glucuronidase–Tat₅₇₋₄₇ was distributed to 22% and 32% of the brain, respectively. Increased distribution of expressed enzyme after intraventricular injection has important implications for enzyme-based therapies and for gene transfer using vectors with high affinity to the ependymal lining, such as recombinant adenoviruses¹¹ and adeno-associated virus type 4 (ref. 12).

Earlier studies demonstrated that the 11-amino acid protein transduction motif from HIV Tat could be used to improve transfer of synthetic peptides and nuclear or cytoplasmic reporter/fusion

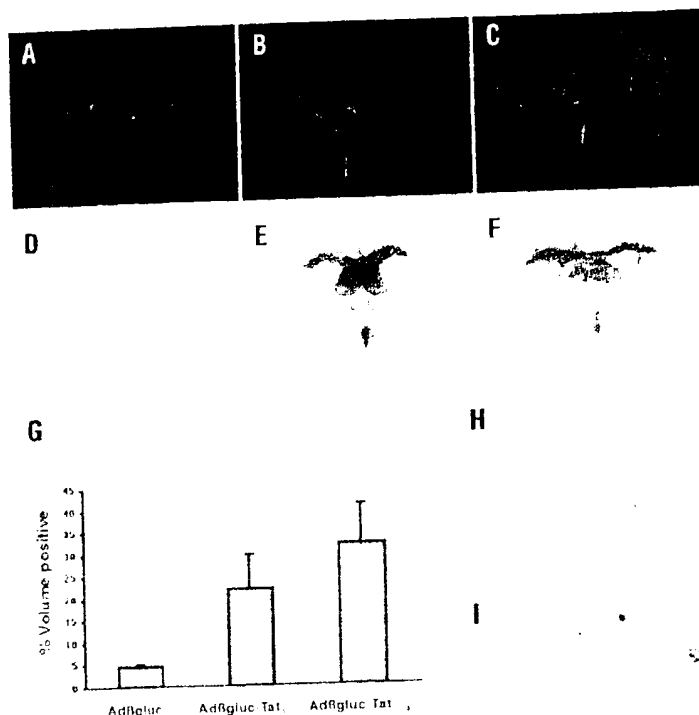


Figure 5. Expression of β -glucuronidase or β -glucuronidase-Tat₄₇₋₅₇ from transduced ependyma. Mice were injected into the right lateral ventricle with Ad β gluc (A,D), Ad β gluc-Tat₄₇₋₅₇ (B,E), or Ad β gluc-Tat₅₇₋₄₇ (C,F), and brains were harvested 10 days later for evaluation of GFP (A-C) or β -glucuronidase (D-F) expression. Sections photomicrographed in panels A-C are within 60 μ m from those shown in panels D-F. (G) Volume of brain (both hemispheres) positive for β -glucuronidase activity was determined using NIH image analysis software. There was a significant increase in the volume of brain positive for β -glucuronidase after Ad β gluc-Tat₄₇₋₅₇ and Ad β gluc-Tat₅₇₋₄₇ vs. Ad β gluc injection ($P < 0.005$). (H) Characteristic lysosomal pathology in the cortex of a 12-week-old β -glucuronidase-deficient mouse. (I) Representative photomicrograph depicting the reduction of lysosomal storage material in the contralateral cortex of age-matched β -glucuronidase-deficient mice five weeks after injection of Ad β gluc-Tat₄₇₋₅₇ into the ventricle. Bar, 10 μ m.

proteins into cells. Our work extends the application of this motif to enzymes specific to the lysosomal compartment and relevant to genetic diseases affecting the viscera and/or brain. The significant improvements in β -glucuronidase distribution imparted by the Tat motif have important implications as we advance gene and protein therapies from mouse models to human applications.

Experimental protocol

Recombinant vectors. Primer 1 (5'-AAACTCGAGATGGCCCGGGG GTCGGCGGTTGCC-3') and primer 2 (5'-TGCTCTAGATCATCTTCG TCGCTGTCTCCGCTTCTTCTCGCCATAACCGCCACCGCCAGTAA CCGGCTGTTTCCAAACA-3') were used to create the β -glucuronidase-Tat₄₇₋₅₇ fusion protein. Primer 1 and primer 3 (5'-TGCTCTA GATCAATAGCCCTCTTCTTCCGCTCTGTCTGCTCTACCGCCAC- CGCCAGTAAACGGGCTGTTTCCAAACA-3') were used to make the β -glucuronidase-Tat₅₇₋₄₇ fusion protein. PCR fragments were digested with *Xho*I and *Xba*I, and the fragments were cloned into similarly cut E1 shuttle plasmids (pPacRSV β Gluc-Tat PTD₄₇₋₅₇ or pPacRSV β Gluc-Tat PTD₅₇₋₄₇). Adenoviruses containing two distinct expression cassettes, β -glucuronidase, β -glucuronidase-Tat PTD₄₇₋₅₇, or β -glucuronidase-Tat PTD₅₇₋₄₇ in E1, and enhanced GFP in E3 were produced by homologous recombination in

human embryonic kidney (HEK) 293 cells. Briefly, PacI-linearized pPacRSV β Gluc-Tat PTD₄₇₋₅₇, pPacRSV β Gluc-Tat PTD₅₇₋₄₇, or pPacRSV β Gluc was cotransfected along with PacI-digested E3 modified Ad5 backbones containing a RSVGFP expression cassette in E3 (H. Xia and B.L. Davidson, unpublished data). For ease of discussion, the recombinant viruses, Ad β gluc-Tat₄₇₋₅₇/E3GFP, Ad β gluc-Tat₅₇₋₄₇/E3GFP, or Ad β gluc/E3GFP are listed as Ad β gluc-Tat₄₇₋₅₇, Ad β gluc-Tat₅₇₋₄₇, or Ad β gluc. Viruses were purified by CsCl gradient ultracentrifugation. Infectious units were determined by plaque assay and particle titers by OD₂₆₀.

In vitro studies. HeLa cells were infected with Ad β gluc-Tat₄₇₋₅₇, Ad β gluc-Tat₅₇₋₄₇, or control Ad β gluc at 20 i.u./cell, and supernatants were harvested 72 h later. β -Glucuronidase activity was quantified using the previously described fluorometric assay. Briefly, aliquots were reacted in 10 mM 4-methylumbelliferyl- β -D-glucuronide (Sigma, St. Louis, MO) in 0.1 M sodium acetate (pH 4.8) for 1 h at 37°C. Reactions were stopped by addition of 2 ml of 320 mM glycine in 200 mM carbonate buffer at pH 10.0 (ref. 14). Fluorescence was measured at 415 nm after excitation at 360 nm (TD-700 Fluorometer; Turner Design, Sunnyvale, CA). β -Glucuronidase activity is expressed as nanomoles of 4-methylumbelliferone released per hour (fluorometric units, FU) per milligram protein. Purified β -glucuronidase (kindly provided by William Sly, Washington University, St. Louis, MO) was used as standard. Protein concentrations were determined using the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA).

Cells (NIH3T3 or A549; 500,000 cells plated the day before) were incubated with 5,500 units of β -glucuronidase-Tat₄₇₋₅₇, β -glucuronidase-Tat₅₇₋₄₇, or β -glucuronidase in the presence or absence of D-M6P (10 mM) for 2 h at 37°C or 4°C. After incubation, cells were harvested and lysates were prepared for fluorometric enzyme assay or stained for β -glucuronidase activity *in situ*. For β -glucuronidase staining, cells were washed in PBS, fixed in 2% paraformaldehyde for 15 min, washed twice in PBS, twice with 0.05 M sodium acetate, pH 4.5, for 5 min, and then incubated in 0.25 mM Naph-As-Bi- β -glucuronide (Sigma) in the same buffer for 40 min. Cells or tissues (below) were then stained for 30 min at 37°C with 0.25 mM Naph-As-Bi- β -glucuronide in 0.05 M sodium acetate, pH 5.2, with 1:500 2% hexazotized pararosaniline (Sigma). One-way analysis of variance was used to test the effect of M6P on the entry of β -glucuronidase or Tat-modified β -glucuronidase into cells.

In vivo studies. β -Glucuronidase-deficient mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and from our own breeding colony. The genotype for the latter was confirmed by morphological and genetic analyses. For the evaluation of correction for storage pathology in the spleen, the mice were 4.5 weeks old at the time of injection. For brain correction, 7-week-old and 3.5-month-old mice were used. For all other experiments, animals were between 9 and 10 weeks old and weighed 16–24 g. C57BL/6 wild-type mice were purchased from Harlan Sprague (Indianapolis, IN). All animal procedures were approved by the University of Iowa Animal Care and Use Review Committee.

For enzyme activity assay and *in situ* staining, Ad β gluc-Tat₄₇₋₅₇, Ad β gluc-Tat₅₇₋₄₇, or Ad β gluc was injected into the tail vein (2×10^9 i.u.) of β -glucuronidase-deficient mice, Ad β gluc-Tat₄₇₋₅₇, Ad β gluc-Tat₅₇₋₄₇, or Ad β gluc (2×10^7 i.u. total) were injected into the right striatum or right lateral ventricle of C57BL/6 mice or β -glucuronidase-deficient mice as described earlier¹⁰. Animals were killed 10 days after intravenous ($n = 3$ /group), striatal ($n = 5$ /group), or ventricular ($n = 5$ /group) injection. Tissues were sonicated, placed in lysis solution¹⁵, and centrifuged at 12,000 g for 20 min. Aliquots were assayed using the fluorometric assay described above. For *in situ* enzyme assays, tissues were harvested, sectioned, and stained *in situ* for β -glucuronidase activity as described above. Coronal brain sections were photographed with Adobe Photoshop (Adobe System, Mountain View, CA), and the photos were imported into National Institutes of Health (NIH) image analysis software. Color thresholding was used, and the percentage of brain positive for activity was calculated by dividing the area of staining by

the total area (adjusted for ventricular size). In all cases, a minimum of 2 mm (rostral to caudal) of cerebrum surrounding the injection site was scanned. Enzyme assay and volumetric data were evaluated by one-way analysis of variance followed by pairwise comparison of the means using Bonferroni's method. For evaluation of correction of histology, animals were killed 18 days after tail vein injection ($n = 4$ samples) or 5 weeks after intraventricular injection ($n = 3$). Tissues were processed through 2% paraformaldehyde/2% glutaraldehyde and embedded in Spurr's medium as described¹⁰.

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